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Development of Luminescent *Mycobacterium avium* subsp. *paratuberculosis* for Rapid Screening of Vaccine Candidates in Mice

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***Mycobacterium avium* subsp. *paratuberculosis* is a slowly growing mycobacterial species, requiring 6 to 8 weeks of culture before colonies can be counted visually. Here, we describe the development of luminescent *M. avium* subsp. *paratuberculosis* expressing *luxAB* genes of *Vibrio harveyi* and its use for vaccine testing in an experimental mouse model, replacing fastidious CFU counting by rapid luminometry.**

Experimental *Mycobacterium avium* subsp. *paratuberculosis* infection and vaccination studies are seriously hampered by technical problems. *M. avium* subsp. *paratuberculosis* is a slowly growing mycobacterial species, requiring 6 to 8 weeks of culture before colonies can be counted visually. Determining the number of CFU in organ homogenates requires labor-intensive plating on expensive mycobactin-supplemented Middlebrook agar (5). Here, we report on the construction of two luminescent *M. avium* subsp. *paratuberculosis* isolates: i.e., reference strain ATCC 19698 (7) and strain S-23, a clinical isolate of bovine origin (4). We show, in an experimental mouse model, that rapid and cheap luminometry can replace fastidious CFU enumeration on agar, and furthermore we demonstrate that luminescent *M. avium* subsp. *paratuberculosis* ATCC 19698 can be used for the rapid screening of potential new paratuberculosis vaccine candidates.

We have previously reported on the use of pYUB180-transformed *M. avium* subsp. *paratuberculosis* strain K-10 encoding *Photinus pyralis* luciferase for antimicrobial drug susceptibility testing (12). However, attempts to use this firefly luciferase-expressing K-10 isolate for in vivo testing in an experimental mouse model were unsuccessful, because of the low sensitivity of the Turner Design 20/20 luminometer (10) (our unpublished data). Based on our earlier experience with luminescent *Mycobacterium tuberculosis* H37Rv (3, 11), encoding bacterial *luxAB* genes from *Vibrio harveyi*, we decided to use the same approach for *M. avium* subsp. *paratuberculosis*. *M. avium* subsp. *paratuberculosis* reference strain ATCC 19698 and strain S-23 were transformed as described previously (12) with plasmid pSMT1 encoding *luxAB* genes downstream from the *Mycobacterium bovis* BCG *hsp60* promoter and a hygromycin resistance gene as a selectable marker (10). pSMT1 DNA was prepared in *Escherichia coli* with a Wizard Miniprep kit (Promega, Madison, WI). Transformants were grown at 37°C for 5 weeks on Middlebrook 7H9 agar supplemented with OADC (oleic acid, albumin, dextrose, and catalase), mycobactin J (Allied Laboratories Inc., Synbiotics Europe) (2 μg/ml), and 50 μg/ml hygromycin. This is the first report on the use of this drug marker to select *M. avium* subsp. *paratuberculosis* transformants. Transformed, luminescent *M. avium* subsp. *paratuberculosis* ATCC 19698 or S-23 was grown in Middlebrook 7H9 medium supplemented with OADC, mycobactin J, and hygromycin, to an optical density of 0.6. Bacteria were washed in phosphate-buffered saline (PBS), and the number of bioluminescent bacteria was determined using a bioluminescence assay in a Turner Design 20/20 luminometer with 1% *n*-decyl-aldehyde (Sigma) and ethanol as a substrate (11). Compared to a classical firefly luciferase-based test, this bacterial luciferase-based assay has the economical advantage of combining a low-price luminometer with an inexpensive substrate. In this assay, only live bacteria are enumerated, as light emission is dependent on the presence of reduced flavin mononucleotide ($FMNH_2$), a cofactor only found in living cells. Female BALB.B10 mice (bred at the Animal Facilities of the Pasteur Department) were infected intravenously in a lateral tail vein with 0.2 ml of bacteria, adjusted to 2×10^6 milli-relative light units (mRLU)/ml. The number of CFU of these preparations, determined by plating serial dilutions in PBS on Middlebrook 7H11-OADC agar supplemented with mycobactin J and hygromycin (100 μg/ml), was 5×10^6 CFU/ml. Thus, the CFU/mRLU ratio for axenic *M. avium* subsp. *paratuberculosis* cultures was 2.5. This ratio is a relative value specific for each laboratory and dependent on the type of luminometer used. The number of bacteria in spleen homogenates of individual infected mice was determined 5, 10, and 15 weeks after infection. Mice were killed by cervical dislocation, and spleens were removed aseptically and homogenized in 10 ml of PBS using a loosely fitting Dounce homogenizer (8). For luminometry, fresh 1-ml spleen homogenates were tested in duplicate after erythrocyte lysis (to minimize quenching) as described previously (11). For CFU plating, 100-μl volumes of serial dilutions of spleen homogenate in PBS were plated in duplicate on Middlebrook 7H11-OADC agar supplemented with mycobactin J. To check for the presence or loss of the pSMT1 plasmid, platings were performed in media with (h) or without hygromycin (100 μg/ml). Petri dishes were sealed in plastic bags and incubated at 39°C for 8 weeks before visual counting. For all statistical analyses (Student's *t* test), luminometry results obtained in mRLU and plating re-

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TABLE 1. Bacterial replication of luminescent *M. avium* subsp. *paratuberculosis* ATCC 19698 and S-23 in spleens from BALB.B10 mice, analyzed 5, 10, and 15 weeks postinfection[a]

Wk after infection	Bacterial count[b]					
	ATCC 19698			S-23		
	Log_{10} mRLU	Log_{10} CFU (h)	Log_{10} CFU	Log_{10} mRLU	Log_{10} CFU (h)	Log_{10} CFU
5	4.06 ± 0.30	5.61 ± 0.29	5.61 ± 0.26 A	4.01 ± 0.15	5.46 ± 0.14	5.55 ± 0.09 A
10	3.87 ± 0.11 C	5.42 ± 0.19 C	5.44 ± 0.26 AC	4.35 ± 0.27 C	5.59 ± 0.07 C	5.90 ± 0.15 BD
15	3.78 ± 0.07 C	5.42 ± 0.12 C	5.46 ± 0.09 AC	4.31 ± 0.04 D	5.63 ± 0.14 C	6.14 ± 0.21 BD

[a] Spleens from individual infected mice (n = 3/group) were homogenized, and the number of bacteria/spleen was enumerated by luminometry (results in mRLU converted to log_{10}) or by CFU plating on Middlebrook 7H11 agar supplemented with hygromycin (h) or not supplemented.

[b] P values that represent probabilities compared to log_{10} CFU (h) values, calculated using Student's t test, are indicated as follows: A, not significant; B, $P < 0.05$. P values that represent probabilities compared to the number of bacteria on week 5, as calculated using Student's t test, are indicated as follows: C, not significant; D, $P < 0.05$.

sults obtained in CFU were converted to mean log_{10} values/total spleen.

As shown in Table 1, both luminescent *M. avium* subsp. *paratuberculosis* strains could be detected in the spleens of infected BALB.B10 mice by luminometry and CFU plating throughout the entire 15-week follow-up period. Intravenous infection of seven inbred mouse strains with luminescent *M. avium* subsp. *paratuberculosis* showed that genetic susceptibility to *M. avium* subsp. *paratuberculosis* infection was controlled by *Nramp1* (9), with BALB.B10 mice displaying a susceptible phenotype (V. Rosseels et al., unpublished data). The S-23 clinical strain (kept with a low number of in vitro passages) was somewhat more virulent in this mouse model than was the ATCC 19698 strain (dating back to 1979), as both luminometry and CFU plating of S-23 showed a modest increase in bacterial number in the spleens of BALB.B10 mice between weeks 5 and 15 after infection, whereas bacterial numbers of the ATCC 19698 strain remained constant over the 15-week test period. The numbers of CFU of *M. avium* subsp. *paratuberculosis* ATCC 19698 determined with or without hygromycin were identical at the three time points tested, whereas the luminescent S-23 strain showed a tendency to lose the pSMT1 plasmid, resulting in 0.41 and 0.51 log_{10} less CFU at weeks 10 and 15, respectively, in 7H11 agar supplemented with hygromycin than in agar without hygromycin. The CFU/mRLU ratios of these ex vivo-isolated mycobacteria were 35.5 for ATCC 19698 and 34.7 for S-23 after 5 weeks of infection. These ratios were about 15-fold higher than that for in vitro-grown *M. avium* subsp. *paratuberculosis* and can be explained by light quenching effects and by reduced fitness of the bacteria isolated from the harsh environment of the macrophage phagosome. A similar difference in CFU/mRLU ratio has been observed for in vitro-grown and ex vivo-isolated luminescent *M. tuberculosis* H37Rv (K. Huygen, unpublished data). The luciferase-based assay had two advantages over classical CFU plating in addition to its rapidity and inexpensiveness. The luminescence assay on duplicate samples was very reproducible, with 5 to 10% intra-assay variation. CFU counting was less accurate, with intra-

TABLE 2. Protective efficacy of irradiated *M. avium* subsp. *paratuberculosis* and *M. bovis* BCG vaccines, as tested by luminometry and CFU plating of spleen cell homogenates[a]

Wk after infection	No. of bacteria in spleen[b]				
	Unvaccinated	Irradiated *M. avium* subsp. *paratuberculosis*		*M. bovis* BCG	
		Count	Δ[c]	Count	Δ
5					
Log_{10} mRLU	4.06 ± 0.30 (3)	3.44 ± 0.23 (5)*	0.62	3.06 ± 0.08 (3)**‡	1
Log_{10} mCFU (h)	5.58 ± 0.31 (3)	5.21 ± 0.31 (5)†	0.37	5.08 ± 0.23 (3)†	0.50
Log_{10} mCFU	5.54 ± 0.26 (3)	5.22 ± 0.30 (5)†	0.32	5.08 ± 0.17 (3)†	0.46
10					
Log_{10} mRLU	3.87 ± 0.11 (3)	3.22 ± 0.14 (5)***	0.65	3.03 ± 0.07 (3)***	0.84
Log_{10} mCFU (h)	5.47 ± 0.17 (3)	5.13 ± 0.17 (4)*	0.34	4.88 ± 0.28 (3)*	0.59
Log_{10} mCFU	5.49 ± 0.22 (3)	5.24 ± 0.14 (4)†	0.25	4.91 ± 0.31 (3)†	0.58
15					
Log_{10} mRLU	3.78 ± 0.07 (3)	3.57 ± 0.24 (5)†	0.21	3.12 ± 0.08 (4)***‡	0.66
Log_{10} mCFU (h)	5.41 ± 0.26 (3)	4.83 ± 0.30 (5)*	0.58	4.64 ± 0.12 (4)**	0.7
Log_{10} mCFU	5.45 ± 0.18 (3)	4.82 ± 0.36 (4)*	0.63	4.61 ± 0.06 (3)**	0.84

[a] BALB.B10 mice were sacrificed 5, 10, or 15 weeks after intravenous *M. avium* subsp. *paratuberculosis* infection, and the number of bacteria in the spleen was determined in individual spleen homogenates by luminometry (log_{10} mRLU) or CFU plating on Middlebrook 7H11 agar supplemented with hygromycin (h) or not supplemented.

[b] The number of mice is given in parentheses. P values calculated using Student's t test, compared to the number of bacteria in unvaccinated mice, are indicated as follows: †, not significant; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; and ***, $P < 0.001$. ‡, P value of <0.05 compared to the number of bacteria in mice vaccinated with irradiated *M. avium* subsp. *paratuberculosis*.

[c] $\Delta = log_{10}$ mRLU[CFU(h), CFU] unvaccinated − log_{10} mRLU[CFU(h), CFU] vaccinated.

assay variations between 10 to 25%. Also, the detection window for luminometry was much wider, ranging between 10^2 and 10^7 mRLU, whereas precise CFU counting was possible only for 10 to 100 CFU/petri dish, requiring a series of spleen homogenate dilutions.

In order to validate the model for vaccine screening, we applied this luminometric technology to compare the protective efficacies as vaccines of γ-irradiated *M. avium* subsp. *paratuberculosis* ATCC 19698 and the live *M. bovis* BCG vaccine used against bovine (2) and human (1) tuberculosis. *M. avium* subsp. *paratuberculosis* ATCC 19698 was grown for 2 weeks as a surface pellicle on synthetic mycobactin J-supplemented Sauton medium and γ-irradiated with 0.5 megarads (IBA Mediris, Fleurus, Belgium). Female BALB.B10 mice were vaccinated subcutaneously with 0.5 mg (wet weight) of irradiated *M. avium* subsp. *paratuberculosis* ATCC 19698 in Montanide ISA 775 (SEPPIC) adjuvant or intravenously in a lateral tail vein with 0.5 mg ($\pm 2 \times 10^6$ CFU) of BCG and challenged 20 weeks later by the intravenous route with 0.2 ml of luminescent *M. avium* subsp. *paratuberculosis*. The challenge was performed with the reference ATCC 19698 strain (4×10^5 mRLU/mouse), which had demonstrated 100% stability of its pSMT1 plasmid. As shown in Table 2, BALB.B10 mice immunized with irradiated *M. avium* subsp. *paratuberculosis* vaccine or live *M. bovis* BCG demonstrated significantly reduced bacterial counts after challenge compared to unvaccinated mice. BCG vaccine appeared to be slightly more effective than irradiated *M. avium* subsp. *paratuberculosis*, but the difference was statistically significant in luminometry only at week 5 and week 15 after challenge. At the first two time points after challenge, luminometry was more sensitive for detecting vaccine efficacy than for CFU counting. Thus, at week 5 after challenge, BCG vaccine resulted in a 10-fold reduction (1 $\log_{10}$) in mRLU counts, whereas CFU values were reduced only threefold (0.5 $\log_{10}$). This difference between the two assays could not be explained by loss of plasmid, as there was no difference in the numbers of bacteria grown with or without hygromycin. Comparing mRLU and CFU (h) values in unvaccinated mice, CFU/mRLU ratios of 33, 40, and 42 were found at 5, 10, and 15 weeks after infection, respectively. For mice vaccinated with irradiated *M. avium* subsp. *paratuberculosis*, these differences were 59, 81, and 18; and finally for mice vaccinated with live BCG, CFU/mRLU ratios were 105, 71, and 33. These different CFU/mRLU ratios in vaccinated and unvaccinated mice suggest that at least at the first two time points, the overall fitness of the bacteria was more impaired in the vaccinated than in the unvaccinated groups. We hypothesize that the luciferase-based assay could actually detect not only differences in numbers of bacteria but also differences in their metabolic activity. The latter would result in bacterial colonies of smaller sizes, which is technically very difficult to demonstrate for *M. avium* subsp. *paratuberculosis*, in view of its extremely low multiplication rate.

In conclusion, our results show that luminescent *M. avium* subsp. *paratuberculosis* transformed with pSMT1 plasmid encoding the *luxAB* genes of *Vibrio harveyi* may be a valuable tool for the rapid, simple, and cheap in vivo screening of vaccine candidates. Moreover, these luminescent bacteria could also be used for in vitro drug testing and in vitro monitoring of bacterial replication in cell lines such as bovine epithelial cells.

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